0: 15'00 M24 - AH

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: 0 260 880 A2

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 87308016.2

(22) Date of filing: 10.09.87

(51) Int. Cl.⁴: A61K 35/12 , A61K 39/395 , C12N 15/00 , C12P 21/00

The microorganism(s) has (have) been deposited with American Type Culture Collection under number(s) ATCC 40268.

(30) Priority: 11.09.86 US 906413
18.11.86 US 932871

(43) Date of publication of application: 23.03.88 Bulletin 88/12

(84) Designated Contracting States: BE DE FR GB IT NL SE

DOC

(71) Applicant: DANA-FARBER CANCER INSTITUTE, INC.
44 Binney Street
Boston Massachusetts MA 02115(US)

—INF C12 N15/12

(72) Inventor: Reinherz, Ellis L.
113 South Great Road
Lincoln Massachusetts 01773(US)
Inventor: Siliciano, Robert F.
99 Marion Street
Brookline Massachusetts 02146(US)
Inventor: Sayre, Peter
Two Peabody Terrace Apt. 406
Cambridge Massachusetts 02138(US)
Inventor: Chang, Hsiu-Ching
16 Lowell Street
Cambridge Massachusetts 02138(US)
Inventor: Richardson, Neil
367 Hunnewell Street
Needham Heights Massachusetts 02194(US)

(74) Representative: Field, Roger Norton et al
Brewer & Son 5-9, Quality Court Chancery Lane
London WC2A 1HT(GB)

(54) Turning on of cytotoxicity.

(57) The invention provides a method of turning on the cytolytic effector function of human cytolytic T-cells or human natural killer cells, the method involving contacting the cells with a substance or mixture of substances characterized in that the substance or the mixtures binds specifically to the T11 sheep erythrocyte binding glycoprotein of the cells and is capable upon the binding of turning on the cytolytic effector function. Also provided is a cDNA sequence encoding human T11 or a fragment thereof which is capable of inhibiting T-lymphocyte activation.

M07K201:00 –
M07K209:00 –
M07K207:00 –

which permit the cell-to-cell contact necessary for lymphocyte proliferation. To test a fragment for the ability to inhibit lymphocyte proliferation, or the cytotoxic effector function, the fragment is contacted with the lymphocytes prior to stimulation with mitogen, and degree of proliferation is measured, using standard techniques, and the result compared to a control in which the fragment was not used.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Fig. 1 is a histogram showing lysis of B-lymphoblastoid target cell lines by T-cells treated with various monoclonal antibodies (αT11);

Fig. 2 is a histogram showing the effect of anti-T11 antibodies on the cytolytic effector function of various cell lines;

Fig. 3 is a histogram showing lysis of various target cells by T-cell clones QQ and JT3;

Fig. 4 is a diagrammatic representation of the strategy used in sequencing the cDNA (PB1) encoding T11;

Fig. 5 is the sequence of the PB1 cDNA, with the deduced amino acid sequence of T11 given on the bottom line; and

Fig. 6 is a diagrammatic representation of the T11 molecule, showing functional domains.

Purification and Characterization of T11

4×10^{10} Jurkat cells were washed twice in serum-free medium and lysed for one hour at 4° C in 420 ml in 10mM Tris, pH 8.0 containing 0.15 M NaCl, 1% Triton-X 100, 1 mM iodoacetamide and the following protease inhibitors (Sigma): phenylmethylsulfonylfluoride (1mM), chymostatin (0.5 µg/ml), pepstatin (0.5 µg/ml), antipain (0.5 µg/ml), leupeptin (0.5 µg/ml), trypsin inhibitor (0.02 µg/ml). The crude lysate was centrifuged at 3000 $\times$ g for 20 min. The supernatant was made 0.5% in sodium deoxycholate and ultracentrifuged for 60 min at 150,000 $\times$ g. 25×10^6 Jurkat cells from the same culture were surface radiolabelled by lactoperoxidase-catalyzed iodination. 2×10^7 radiolabelled cells were treated with 0.5 ml of lysis buffer and added to the large scale lysate. The combined lysates were applied at 0.75 ml/min to a 15 ml pre-clear column containing irrelevant mouse monoclonal antibodies anti-T3 (8C8), anti-Ti_3 (9H5) and anti-β2 microglobulin coupled to protein A Sepharose beads CL-4B (Pharmacia) at 5 mg antibody per ml of beads, followed by a 5 ml specific antibody column containing anti-$T11_1$ (8B5) coupled to protein A Sepharose at 5 mg/ml. The anti-T11 column was washed with 10mM Tris, pH 8.0 with or without detergents and eluted in 1 ml fractions with 0.1M glycine, PH 3.0, 0.5% Triton-X 100. The fractions were collected in tubes containing 60 µl 1M Tris pH 8.0.

Fractions containing radioactivity were pooled, made 10% in glycerol and 2% in SDS, heated at 60° C for 20 min, loaded onto a 10% preparative polyacrylamide gel in a single lane 10 cm wide, and electrophoresed for 16 h at 40 volts. A 0.5 cm wide strip of the gel was dried and autoradiographed and the rest stained with Coomassie blue. Stained bands containing T11 were localized by comparison with the migration of surface labelled T11 as shown by the autoradiographed strip. Three regions of the gel at approximately 55, 53 and 50KD were excised. Gel slices were washed with H_2O and electroeluted in 50mM ammonium bicarbonate containing 0.1% SDS for 16 h at 50 volts (Hunkapiller et al., 1983). Sample eluates were collected and proteins precipitated at -20° C for 16 h by the addition of 9 volumes of cold ethanol. Precipitated proteins were collected by centrifugation and the protein pellets vacuum dried.

The ethanol precipitates were resuspended in 0.1% SDS and proteins were sequenced by Edman degradation on a gas phase protein sequenator (Applied Biosystems, model 120A) using the 03RPTH program and aqueous trifluoroacetic acid conversion chemistry. The PTH amino acids were identified with an on-line PTH analyzer (Applied Biosystems, model 120A) using a narrow-bore C18 reverse phase column run in acetate buffered 5% aqueous tetrahydrofuran and developed with acetonitrile. 5-10 µg from the 55, 53 and 50 KD bands were analyzed and an identical N-terminal sequence was obtained for each. 18 of the 19 N-terminal positions were assigned as follows:

```
1                 10              18
K E I T N A L E T XXX G A L G Q D I N
```

Table 1

Cytolytic T-cell	Conditions	Anti-T11$_2$+ Anti-T11$_3$	%Specific ^{51}Cr release Laz 509	Laz 156
QQ	Control	-	6	81
QQ	Control	+	47	68
QQ	5 mM EGTA	-	0	1
QQ	5 mM EGTA	+	2	7
QQ	25°C	-	2	42
QQ	25°C	+	3	5
None	Supernatant*	-	1	0
QQ	Anti-T8	-	6	9
QQ	Anti-T8	+	50	54

*Supernatant of clone QQ triggered with anti-T11$_2$ and anti-T11$_3$ at final dilutions of 1:00 each for 4 h.

Referring still to Table 1, the lysis of Laz 509 and Laz 156 by T-cell clone QQ was measured in the absence and presence of anti-T11 antibodies in the indicated conditions. Anti-T11 antibodies were used in ascites form at a 1:100 final dilution. In one set of wells, QQ cells were omitted and replaced with cell-free supernatants of clone QQ triggered with anti-T11$_2$ and anti-T11$_3$ for 4 h. The supernatants were used at a 50% final concentration.

Calcium is known to be required in the lethal hit stage of antigen-specific cytotoxicity. We found that it is also required for anti-T11-induced cytotoxicity because lysis is almost completely inhibited by EGTA. In addition, both antigen-specific and anti-T11-induced cytotoxicity are inhibited to some extent by low temperature; anti-T11 antibodies are almost completely inhibited at 25°C, whilst specific killing of Laz 156 by QQ is reduced by ~50%. No stable cytolytic factor was detected in the supernatants of T-cell clones triggered with anti-T11$_2$ plus anti-T11$_3$ in this assay system. Consequently, it is likely that either cell contact or close effector-target cell proximity is required for anti-T11-induced cytotoxicity.

These mechanistic similarities between antigen-specific and anti-T11-induced killing suggest that T-cells damage target cells in the same way whether activated through the T3 antigen receptor pathway or via the T11 molecule. Although T11 triggering induces lymphotoxin (LT) secretion by T-cell clones, it is unlikely that anti-T11-induced cytotoxicity is mediated by LT alone because the anti-T11-induced lytic effect can be measured in a 4-h assay using a variety of target cells, including those which are resistant to the effects of LT. In contrast, the detection of LT-mediated cytotoxicity generally requires sensitive target cell lines, longer assays and prior target cell conditioning.

The vast majority of human Natural killer (NK) cells, like early thymocytes, lack Ti α-chain messenger RNA, and consequently express no surface T3-Ti complex. On the other hand, the 50 kD T11 glycoprotein is found on the surface of most NK cells. These results suggest that certain NK cells may be related to T-lineage precursors.

Referring to Fig. 3, various target cells were incubated with T_c and NK clones in the absence (a) or presence (b) of anti-T11$_2$ and anti-T11$_3$. In the absence of monoclonal antibodies, the representative T11$^+$T3$^-$NK clone JT3 efficiently kills the NK-sensitive target cell K562 but not peripheral blood lymphocytes of either of the two B-lymphoblastoid target lines tested. This specificity pattern is clearly distinct from that of the Laz 156-specific T-cell clone QQ. However, after incubation with anti-T11$_2$ and anti-T11$_3$ antibodies, both JT3 and QQ kill all of the target cells to varying degrees. Similar data were obtained for the independently derived NK cell clone JT$_{B18}$ (Hercend et al., 1983, 301 Nature 158). This ability of anti-T11 antibodies to induce killing activity from cells that lack a T3-Ti antigen/MHC receptor complex suggests that the T11 molecule represents an alternative pathway of cell activation. More importantly, these findings suggest that the T11 molecule is a critical structure for inducing cytolytic function in NK cells as well as T-cells.

Referring to Fig. 5, the 1.6Kb insert of PB1 (solid line) was separated from the plasmid by BamHI digestion and subcloned into the M13 sequencing vector mp18. The M13 universal primer was used to derive initial sequence at the 5′ end. Subsequently, primers of 17 nucleotides were used for dideoxy sequencing. Sequencing reactions were also performed on M13 clones which contained BamH I-Sac I and Sac I-BamH I fragments as shown. The open reading frame of the insert is identified by the thick solid bar.

Referring to Fig. 6, there is shown the cDNA and predicted protein sequence of PB1. The probable signal peptide (——), the NH_2-terminus of the mature protein (⟶) and the position of a CNBr cleavage derived fragment (⟹) are shown. Polyadenylation signals at nucleotides 1103 and 1505 are underlined. The last nucleotide before the poly(A) tail in clone PB2 is indicated by an arrowhead at position 1125.

The complete nucleotide sequence of clone PB1 is 1,522 bases in length and flanked by a poly(A) sequence at its 3′ end. An open reading frame of 1,080 bases (positions 24-1103) begins with an ATG methionine codon and is flanked by 23 nucleotides of 5′ untranslated sequence and by 419 bases of 3′ untranslated sequence. A polyadenylation signal (AATAAA) is located 18 bases upstream from the beginning of the poly(A) tail (Fig. 6). PB2 is identical to PB1 except that it lacks four nucleotides present at the 5′ end of PB1 and has a shorter 3′ untranslated region. A poly(A) tail was noted in PB2 after the nucleotide corresponding to residue 1125 in PB1 (arrow head). The bases from positions 1102 to 1103 form part of a Gln codon at amino acid 336 and a stop codon as well as the first five bases of the polyadenylation signal AATAAA (nucleotides 1102-1106) for mRNA corresponding to clone PB2.

The N-terminal lysine of the mature polypeptide is preceded by a sequence coding for a stretch of 24 hydrophobic amino acids which likely represents the signal sequence required for the T11 precursor to be transported across the endoplasmic reticulum. The cDNA sequence predicts three potential N-linked glycosylation sites (Asn-X-Ser/Thr) on the mature protein at amino acid positions 65, 117 and 126. An extremely hydrophobic stretch of 25 amino acids characteristic in size and composition of a transmembrane domain is found at positions 186-210. This region is followed by seven basic amino acids within the next ten residues consistent with the notion that this is the start of the intracytoplasmic domain. 21% of residues in the region of amino acids 211-336 are prolines. This cDNA sequence predicts a molecular weight for the mature polypeptide backbone of 37,994 daltons.

To determine the molecular weight of the broad 50-55KD band in the absence of N-linked sugars, surface labelled T11 was digested with endoF and analyzed by SDS-PAGE. After digestion with endoF, there is a loss of the 55KD band and the appearance of a major component at approximately 40KD. These data suggest that the 55KD protein exists as a 40KD structure in the absence of any N-linked sugar moieties and is consistent with the above determined molecular weight of the protein. These results are consistent with the possibility that all three N-linked glycosylation sites may be utilized.

Two related T11 mRNA species

Northern analysis using polyA+ RNA or cytoplasmic RNA from a variety of sources indicates that expression of the PB1 and PB2 sequences is T lineage specific and yields two common bands of 1.7 and 1.3Kb. Thus, each of six human T lineage cells tested including activated T helper clones, thymus derived tumors, normal thymocytes, activated T cells, and resting peripheral blood T lymphocytes expresses the 1.7 and 1.3Kb transcripts. In contrast, non-T lineage cells such as normal peripheral blood B cells and macrophages, an EBV-transformed B lymphoblastoid line, Laz 509, and the non-lymphoid hematopoietic cell lines HL-60 and U937 lack both transcripts. Given the similarity in size differences between the PB1 and PB2 inserts (~400 bases) and the two species of mRNA, it is likely that PB1 and PB2 cDNAs correspond to the 1.7Kb and 1.3Kb transcripts, respectively. This is further supported by the finding that an oligonucleotide based on a sequence from the 3′ untranslated region unique to PB1 selectively hybridizes in northern analysis to sequences in the 1.7Kb site region.

Considerable variability exists within the level of expression of the 1.7 and 1.3Kb transcripts among individual T lineage tumor cells and physiologic T cell populations. Also, activated T cells express >10 fold higher amounts than resting T lymphocytes. This result is consistent with data indicating that T11 surface expression increases from 20,000 to 200 000 copies per cell upon activation with mitogens or antigens during a six day period and suggests that this differential expression is at least in part transcriptionally regulated.

Cells transfected with PB1, like T-cells which naturally produce T11, do not secrete T11, but rather retain the T11 molecule by means of the transmembrane anchor, with only the external domain exposed. A truncated T11 molecule, with the transmembrane and cytoplasmic domains deleted, can, unlike the complete T11 molecule, be secreted by transfected cells and used in the applications described below. Such a truncated molecule can be prepared in a manner analogous to the method by which an anchor-minus IL-2 receptor molecule was prepared by Treiger et al. (1986) J. Immunol. 136, 4099. The truncated IL-2 receptor was found to be capable of binding to its ligand, IL-2.

To make the truncated T11 molecule, PB1 or PB2 cDNA representing the gene for the entire T11 molecule will be restricted with Pvu II. This enzyme uniquely cuts within the 1,522 base pair T11 molecule cDNA insert at base 629, resulting in removal of all transmembrane and intracytoplasmic sequences and seven amino acids of the external domain. Subsequently, a 14 base phosphorylated synthetic oligonucleotide (CTAAGAATTCTTAG) containing the third base of the codon for amino acid 178, a termination codon TAA followed by the six base recognition sequence for EcoR I (GAATTC), and four nucleotides (TTAG) complementary to CTAA, will be ligated to the Pvu II restricted plasmid DNA with T4 DNA ligase. The DNA will then be digested with Pst I to separate the 5' end of the cDNA insert from the plasmid DNA and then subcloned into an appropriate expression vector. For example, this fragment could be blunted by T4 DNA polymerase to remove the Pst site and then be ligated to the EcoR I linker by T4 ligase. Finally, it could be digested with EcoR I before ligation into the unique EcoR I site of the publicly available PcEXV-1 expression vector.

Use

Human NK and T_c cells can be activated via the T11 molecule in vitro or in vivo and used in the treatment of any medical condition characterized by the presence of unwanted cells. In particular, the method can be used to turn on the cytotoxicity of T_c and NK cells so that they will attack and kill pathogen-infected cells, e.g., cells infected with bacterial, fungal, viral, or protozoan pathogens; or tumor cells, e.g., lung, colorectal, or esophageal cancers.

The first step in in vitro activation is to obtain resting NK and/or T_c cells, either from the patient or a suitable donor. This is typically done by separating out lymphocytes from blood and then, optionally, culturing the lymphocytes in the presence of Interleukin-2 to expand their numbers. (The methods by which lymphocytes are separated out, cultured, treated with Interleukin-2, and used to treat cancer patients are described in detail in Rosenberg et al. (1985) New Eng. J. Med. 313, 1485.)

The lymphocytes and/or NK cells are incubated with the cytotoxicity-inducing substance for a relatively short time period (e.g., four hours at 30-40°C, in the presence of calcium ions), and subsequently infused into the patient. Infusion can be via an arterial or venous catheter or into a large peripheral vein.

Alternatively, rather than activating T_c and NK cells in vitro, the activating substance can be administered in vivo, most preferably by direct perfusion of the tumor with the substance, e.g., via the hepatic artery to induce cytotoxicity of existing NK and T_c Cells.

The methods of the invention induces cytotoxicity in a way which bypasses the normal T11 recognition mechanism, and enables the resultant activated NK and T_c cells to attack and kill their target cells without further treatment. (NK cells have a broad range of target specificity, while T_c cells recognize tumor cells by virtue of tumor-specific antigens.) Unlike treatment with IL-2 alone, treatment according to the invention will activate the totality of NK and T_c cells, and be complete in a few hours.

The T11 protein, or its truncated, secreted form, can be used in a variety of diagnostic and therapeutic applications, all of which are based on the binding of T11 to its natural ligand on human lymphocytes and homologous surface structures present on target cells which facilitate T lymphocyte - target cell interactions, which result in target cell lysis or lymphocyte proliferation. (Siliciano et al. Nature 317:428-431 (1985); and Palacios and Martinez-Maza. J. Immunol. 129:2479-2485 (1982).

The T11 molecule is expressed on the surface of many human T-cell malignancies, e.g., T-cell leukemias and lymphomas. In addition autoimmune diseases, e.g., rheumatoid arthritis and Systemic Lupus Erythmatosis (SLE), are characterized by the presence in the blood and lymph of large numbers of T11-bearing T-cells. Rapid cell turnover in these disease states can cause the shedding of the T11 molecule into the bloodstream.

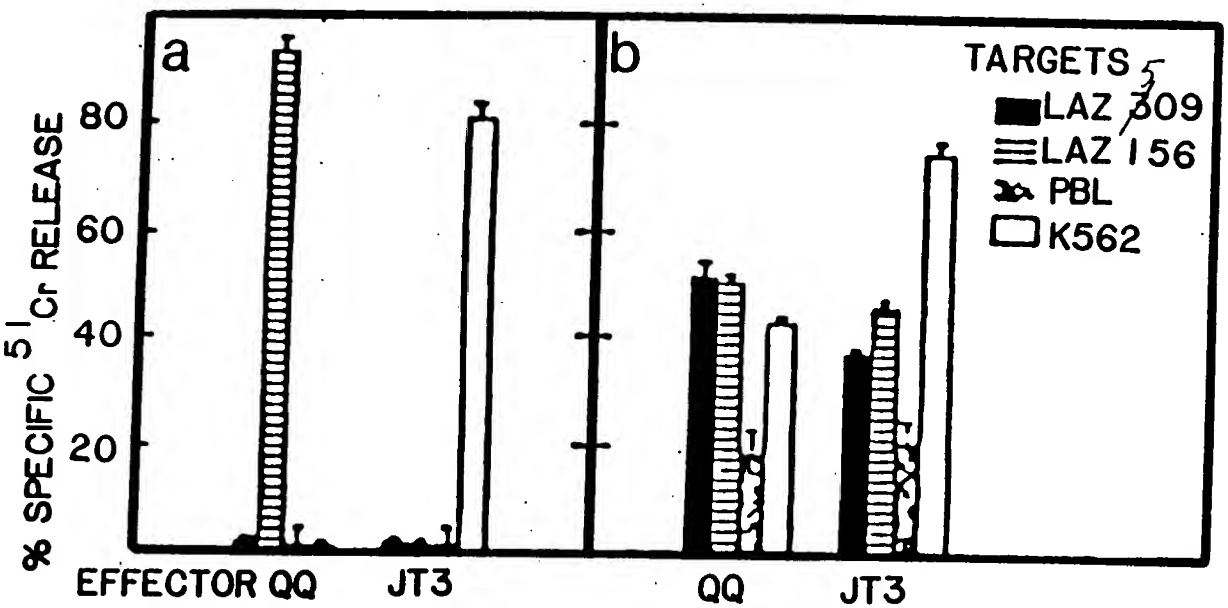
a
b
% SPECIFIC ^{51}Cr RELEASE
80
60
40
20
EFFECTOR QQ
JT3
QQ
JT3
TARGETS
5
LAZ 309
LAZ 156
PBL
K562

FIG. 1

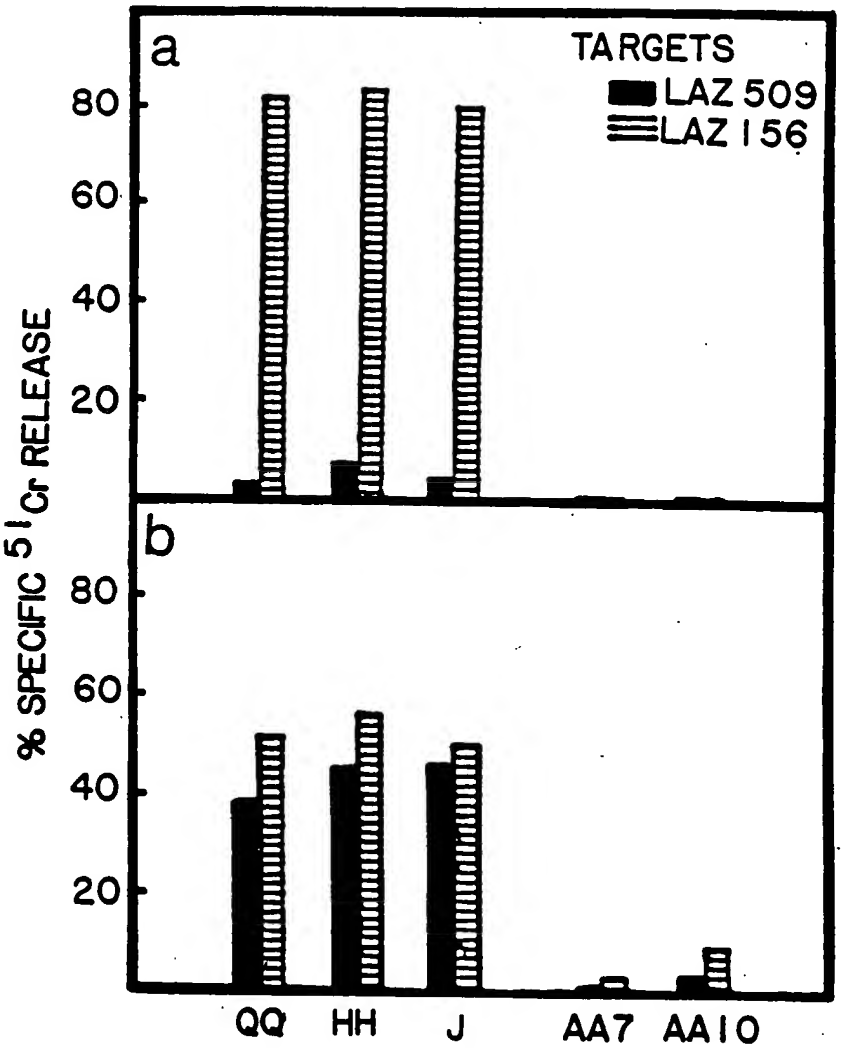
a
TARGETS
LAZ 509
LAZ 156
80
60
40
20
% SPECIFIC ^{51}Cr RELEASE
b
80
60
40
20
QQ
HH
J
AA7
AA10

FIG. 2

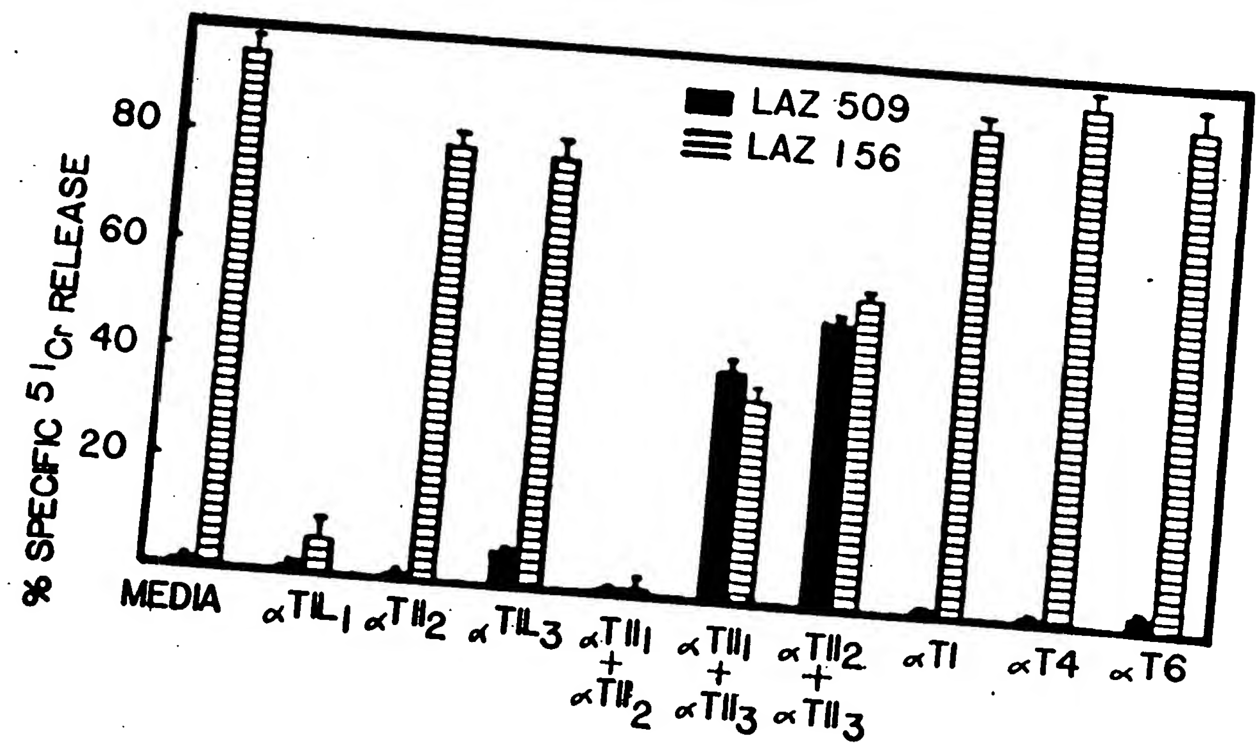
% SPECIFIC 51Cr RELEASE
80
60
40
20
LAZ 509
LAZ 156
MEDIA
αTIL1
αTII2
αTIL3
αTII1 + αTII2
αTII1 + αTII3
αTII2 + αTII3
αTI
αT4
αT6

FIG. 3

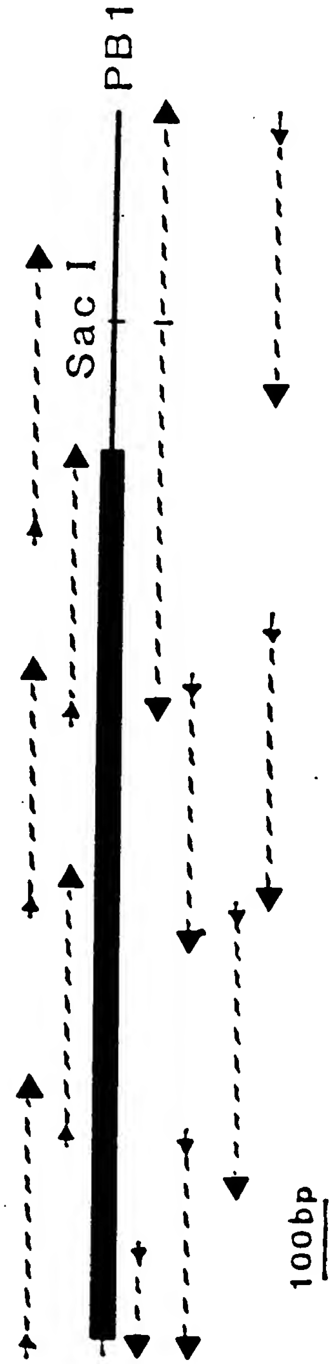

FIG. 4

```
10         20                        38                      53
AAAACAGGAA ACCAACCCCT AAC ATG ACC TTT CCA TGT AAA TTT GTA GCC AGC TTC
                          MET Ser Phe Pro Cys Lys Phe Val Ala Ser Phe      -14

            68                  83                  98
CTT CTG ATT TTC AAT CTT TCT TCC AAA GGT GCA GTC TCC AAA GAG ATT ACC AAT
Leu Leu Ile Phe Asn Val Ser Ser Lys Gly Ala Val Ser Lys Glu Ile Thr Asn      5
                                                    N-term
113             128                 143                 158
GCC TTG GAA ACC TGG GGT GCC TTG GGT CAG GAC ATC AAC TTG GAC ATT CCT AGT
Ala Leu Glu Thr Trp Gly Ala Leu Gly Gln Asp Ile Asn Leu Asp Ile Pro Ser     23

    173             188                 203                     218
TTT CAA ATG AGT GAT GAT ATT GAC GAT ATA AAA TGG GAA AAA ACT TCA GAC AAG
Phe Gln MET Ser Asp Asp Ile Asp Asp Ile Lys Trp Glu Lys Thr Ser Asp Lys     41

            233                 248                 263
AAA AAG ATT GCA CAA TTC AGA AAA GAG AAA GAG ACT TTC AAG GAA AAA GAT ACA
Lys Lys Ile Ala Gln Phe Arg Lys Glu Lys Glu Thr Phe Lys Glu Lys Asp Thr     59

278             293                 308                     323
TAT AAG CTA TTT AAA AAT GCA ACT CTG AAA ATT AAG CAT CTG AAG ACC GAT GAT
Tyr Lys Leu Phe Lys Asn Gly Thr Leu Lys Ile Lys His Leu Lys Thr Asp Asp     77
```

FIG. 5-1

FIG. 5-2

```
338                         353                         368
CAC GAT ATC TAT AAG GTA TCA ATA TAT GAT ACA AAA GGA AAA AAT GTG TTG CAA
Gln Asp Ile Tyr Lys Val Ser Ile Tyr Asp Thr Lys Gly Lys Asn Val Leu Glu    95

383                 398                     413                 428
AAA ATA TTT GAT TTG AAG ATT CAA GAG AGG GTC TCA AAA CCA AAG ATC TCC TGG
Lys Ile Phe Asp Leu Lys Ile Gln Glu Arg Val Ser Lys Pro Lys Ile Ser Trp   113

        443                 458                 473                 488
ACT TGT ATC AAC ACA ACC CTG ACC TGT GAG GTA ATG AAT GGA ACT GAC CCC GAA
Thr Cys Ile Asn Thr Thr Leu Thr Cys Glu Val MET Asn Gly Thr Asp Pro Glu   131
                                                CNBr

                503                 518                 533
TTA AAC CTG TAT CAA GAT GGG AAA CAT CTA AAA CTT TCT CAG AGG GTC ATC ACA
Leu Asn Leu Tyr Gln Asp Gly Lys His Leu Lys Leu Ser Gln Arg Val Ile Thr   149

    548                 563                 578                 593
CAC AAG TGG ACC ACC AGC CTG AGT GCA AAA TTC AAG TGC ACA GCA GGG AAC AAA
His Lys Trp Thr Thr Ser Leu Ser Ala Lys Phe Lys Cys Thr Ala Gly Asn Lys   167

            608                 623                 638
GTC AGC AAG GAA TCC AGT GTC GAG CCT GTC AGC TGT CCA GAG AAA GGT CTG GAC
Val Ser Lys Glu Ser Ser Val Glu Pro Val Ser Cys Pro Glu Lys Gly Leu Asp   185

653                 668                 683                 698
ATC TAT CTC ATC ATT GGC ATA TGT GGA GGA GGC AGC CTC TTG ATG GTC TTT GTG
Ile Tyr Leu Ile Ile Gly Ile Cys Gly Gly Gly Ser Leu Leu MET Val Phe Val   203
```

FIG. 5-3

```
713                         728                         743                         758
GCA CTC CTC GTT TTC TAT ATC ACC AAA ACG AAA AAA CAG ACC AGT CGG ACA AAT
Ala Leu Leu Val Phe Tyr Ile Thr Lys Arg Lys Lys Gln Arg Ser Arg Arg Asn      221

                773                         788                         803
GAT GAG GAG CTG GAG ACA AGA GCC CAC AGA GTA GCT ACT GAA GAA AGG GGC CGG
Asp Glu Glu Leu Glu Thr Arg Ala His Arg Val Ala Thr Glu Glu Arg Gly Arg      239

    818                         833                         848                         863
AAG CCC CAC CAA ATT CCA GCT TCA ACC CCT CAG AAT CCA GCA ACT TCC CAA CAT
Lys Pro His Gln Ile Pro Ala Ser Thr Pro Gln Asn Pro Ala Thr Ser Gln His      257

            878                         893                         908
CCT CCT CCA CCA CCT GGT CAT CGT TCC CAG GCA CCT AGT CAT CGT CCC CCG CCT
Pro Pro Pro Pro Pro Gly His Arg Ser Gln Ala Pro Ser His Arg Pro Pro Pro      275

923                         938                         953                         968
CCT GGA CAC CGT GTT CAG CAC CAG CCT CAG AAG AGG CCT CCT GCT CCG TCC GGC
Pro Gly His Arg Val Gln His Gln Pro Gln Lys Arg Pro Pro Ala Pro Ser Gly      293

        983                         998                         1013                        1028
ACA CAA GTT CAC CAG CAG AAA GGC CCG CCC CTC CCC AGA CCT CGA GTT CAG CCA
Thr Gln Val His Gln Gln Lys Gly Pro Pro Leu Pro Arg Pro Arg Val Gln Pro      311

                1043                        1058                        1073
AAA CCT CCC ATG GGG CAG CAG AAA ACT CAT TGT CCC CTT CCT CTA ATT AAA AAA
Lys Pro Pro MET Gly Gln Gln Lys Thr His Cys Pro Leu Pro Leu Ile Lys Lys      329
```

1088 1103 1116 1126 1136 1146
GAT ACA AAC TGT CTT TTT CAA TAA AAAGCACTGT GGATTTCTGC CCTCCTCATG TGCATATCCG 336
Asp Arg Asn Cys Leu Phe Gln

1156 1166 1176 ~~1186~~ 1178 1196 1206 1216
TACTTCCATG ACCTCTTTTC TCTGTGCAGA ACATTGTCAC CTCCTGAGGC TGTCCCCCAC ACCCACCTCT

1226 1236 1246 1256 1266 1276 1286
GCATCTTCCA ACTCAGCCAT GTCGTCAACA TCTCCACTTT TTGGTCTCCT CACAGAGCTC CATCACACCA

1296 1306 1316 1326 1336 1346 1356
CTAAGGACAA GCAATATAAG TCTCATTGCA AGAACTGTAC ACCACCCAGC CACAAATCTT AGAGATTTCT

1366 1376 1386 1396 1406 1416 1426
TGTCCCCTCT CACCTCATGT GTAGATGCCA TAAATCAAGT CATTCCTGTG CCTCGGTCTC ACTACAACCA

1436 1446 1456 1466 1476 1486 1496
CCCTATCTGC TTAACAGACT CTCCACTTTC TTATCTGCCC TCCTCCACAC TTCCCCACCA TCCTCTCAGT

1506 1516
AAAACTCAAA TAAAACCTTT GACTACA

FIG. 5-4

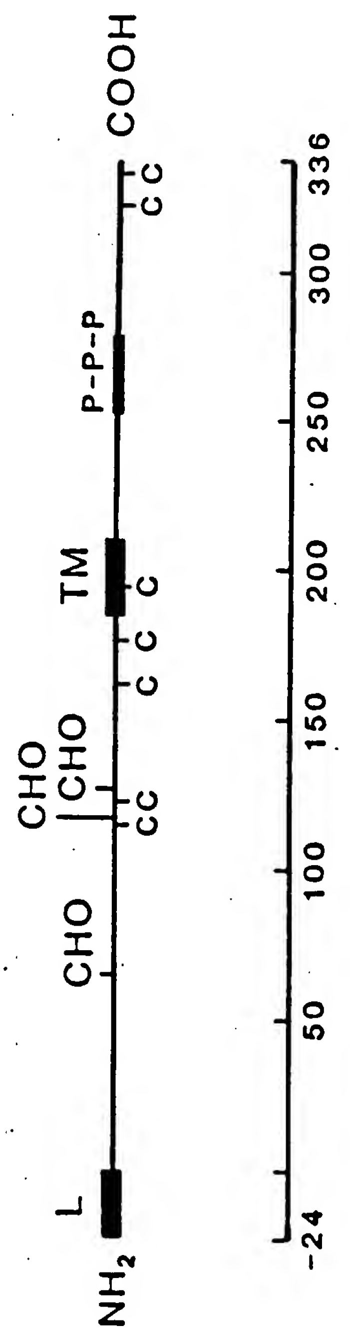
NH2
L
CHO
CHO
CHO
CC
C
C
C
TM
P-P-P
CC
COOH
-24
50
100
150
200
250
300
336

FIG. 6